PLANT GENE CONSTRUCTS AND THEIR USE

The present invention relates to novel plant gene
constructs, and to their use in controlling the flowering
5   of plants. It further relates to plants containing such
constructs.

Plants differ from animals. The adult plant body is formed
post-embryonically by the continuous activity of the shoot
10 and root apical meristems. The shoot apical meristem is
established during plant embryogenesis and together with
cotyledons, hypocotyl, embryonic root and root meristem
makes up the basic body plan.

15 The shoot apical meristem starts as a cluster of about one
hundred cells and is the source of the whole aboveground·
portion of the plant. During the vegetative phase of plant
development this meristem gives rise to (a rosette of)
leaves, stem, and quiescent axillary meristems. This is
20 followed by the formation of secondary inflorescences,
cauline leaves and determinate floral meristems after
floral induction. Flowering involves complex interactions
of gene products that regulate a switch in shoot meristem
identity. Factors determining the expression levels of
25 these genes are genotype and environmental stimuli, such as
photoperiod, temperature and light quality. How the
transition is affected by these stimuli is still largely
unknown.

30 One of the most important events in the plant life cycle is
the decision to enter the reproductive phase. A wide range
of environmental and endogenous signals controls this
transition of the vegetative phase into the reproductive
phase. Important signals are day length, temperature
35 (vernalization), nutrient and water availability and

several phytohormones esp. gibberellin (GA). These signals
induce a shift in vegetative apical meristem identity,
named the floral transition, and this transition
establishes the inflorescence meristem. Whereas the product

5   of the vegetative apical meristem are leaf primordia, the
inflorescence meristem produces primordia that
differentiate into secondary inflorescences during early
generative development and into flowers later in this
stage. In plant breeding research, control of this process

10   is a most important goal for a variety of crops. This is
especially true for rosette plants like lettuce, spinach
and sugar beet, which show rapid stem elongation (bolting)
following the floral transition, and this makes the crop
useless.

15

The transition from vegetative to reproductive growth is a
critical developmental event, and because it is the first
step of sexual reproduction it is of great importance in
agriculture, horticulture, and plant breeding. Farmers may

20   wish to advance or retard the time of flowering, or prevent
it altogether: for example to prevent 'bolting' in e.g.
lettuces or sugar beet. A better understanding of the
molecular biology of plant flowering will allow it to be
controlled or influenced in a number of ways, giving

25   important practical benefits to agriculture.

In PCT Publication WO96/14414, use of the Constans (CO)
gene to modify flowering mechanisms in plants is disclosed.

30   The present invention proposes a way of influencing a
plant's transition from vegetative to reproductive growth,
by providing transformed plants in which the transition is
delayed, or brought forward, by expression of specific
transgenes influencing this process. Such genes may be

35   constitutively expressed, or expressed only in response to

an external stimulus, for example environmental or
chemical.

5   ATH1 is an *Arabidopsis thaliana* homeobox gene. It is
described by Quaedvlieg et al., in Plant Cell 7, 117-129,
1995 (herein incorporated by reference): its DNA sequence
is given in Figure 1 of that paper.   It was isolated from
a light-induced transcription factor collection. It is

10   expressed in young seedlings and flowers. ATH1 mRNA levels
in etiolated seedlings are strongly light-dependent
(phytochrome) and are also light-adaptive.

We have now established that the protein product of ATH1 is

15   involved in the developmental switch from vegetative to
generative growth. As a result of ATH1::GUS studies and'
initial 35S::ATH1 studies, we have deduced that ATH1 has a
function in the transition of the vegetative apical
meristem to an inflorescence meristem. Specifically, ATH1

20   acts as an anti-gibberellin, by repressing GA synthesis or
possibly the GA response pathway: Example 6 illustrates
this.

Our studies on ATH1::GUS constructs have revealed that in

25   young, light-grown seedlings ATH1 is expressed in all three
layers of the shoot apical meristem and leaf primordia. In
young, still developing leaves ATH1 is expressed in
vascular tissue. This expression disappears in developed
leaves. Remarkably, ATH1 meristem expression is restricted

30   to the vegetative phase of development. As soon as
Arabidopsis starts flowering (vegetative to generative
transition) and the shoot apical meristem has become an
inflorescence meristem, ATH1 expression in the meristem is
downregulated. During the inflorescence phase ATH1 is at a

35   low level expressed in developing vascular tissue of the

stem. Later in plant development, when flowers arise, ATH1
is expressed in different parts of the young flower
(receptacle, sepals and vascular tissue of stamen). Our
hypothesis that ATH1 is involved in controlling the phase
5   transition from vegetative to generative growth is further
corroborated by the flowering time phenotypes of ATH1 sense
and antisense over-expressors. Plants ectopically
overexpressing antisense ATH1 show an early-flowering
phenotype: conversely, most plants carrying a sense ATH1
10   overexpression construct are late flowering. A small
proportion of the plants carrying the overexpression
construct are, due to ATH1 reduction by co-suppression,
early flowering, like the antisense ATH1 over-expressors,
and the phenotype of these plants resembles that of the
15   terminal flower mutant(Shannon and Meeks-Wagner, 1991) and
the phenotypes of LEAFY- (Weigel and Nilsson, 1995),
APETALA 1- (Mandel and Yanofsky, 1995) and CONSTANS
(Putteril et al., 1995)over-expressors. Based on these
results, combined with the ATH1::GUS data, we deduce that
20   ATH1 is involved in controlling the phase transition from
vegetative to generative growth in Arabidopsis thaliana,
and probably is a flowering time gene.

In consequence, this transition may be promoted by
25   inhibiting the expression of the ATH1 gene: or retarded or
prevented by promoting such expression.

Accordingly, the present invention provides a plant gene
construct comprising a complete or partial DNA sequence
30   coding for an ATH1 gene product under the control of a
promoter functional in plants. The promoter is preferably
heterologous. The invention further comprises plant cells
transformed with a such a plant gene construct, and plants
comprising such cells having modified flowering properties.
35   The invention further comprises a process for modifying the

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flowering process in plants by transforming plants with a
construct according to the invention.

The use of gene sequences to inhibit or promote gene
5   expression is quite well understood. A complete gene
sequence, under the control of a promoter that operates
effectively in the plant, will generally overexpress the
gene product, leading to an amplification of the effect of
the protein so produced. Sometimes the gene product is
10   reduced: this phenomenon is termed "co-suppression".
Reduction of the gene product is also generally obtained by
using a dominant negative mutation, or by reversing the
orientation of the gene sequence with respect to the
promoter so that it produces "antisense" messenger RNA.
15

        A DNA construct according to the invention may
be an "antisense" construct generating "antisense" RNA or a
"sense" construct (encoding at least part of the functional
protein) generating "sense" RNA. "Antisense RNA" is an RNA
20   sequence which is complementary to a sequence of bases in
the corresponding mRNA: complementary in the sense that
each base (or the majority of bases) in the antisense
sequence (read in the 3' to 5' sense) is capable of pairing
with the corresponding base (G with C, A with U) in the
25   mRNA sequence read in the 5' to 3' sense. Such antisense
RNA may be produced in the cell by transformation with an
appropriate DNA construct arranged to generate a transcript
with at least part of its sequence complementary to at
least part of the coding strand of the relevant gene (or of
30   a DNA sequence showing substantial homology therewith).
"Sense RNA" is an RNA sequence which is substantially
homologous to at least part of the corresponding mRNA
sequence. Such sense RNA may be produced in the cell by
transformation with an appropriate DNA construct arranged
35   in the normal orientation so as to generate a transcript

with a sequence identical to at least part of the coding
strand of the relevant gene (or of a DNA sequence showing
substantial homology therewith). Suitable sense constructs
may be used to inhibit gene expression (as described in
5 International Patent Publication WO91/08299) or a sense
construct encoding and expressing the functional protein
may be transformed into the plant to over-express the
protein.

10 DNA constructs according to the invention may
comprise a base sequence at least 10 bases (preferably at
least 35 bases) in length for transcription into RNA.
There is no theoretical upper limit to the base sequence -
it may be as long as the relevant mRNA produced by the cell
15 - but for convenience it will generally be found suitable
to use sequences between 100 and 1000 bases in length. The
preparation of such constructs is described in more detail
below.

20 As a source of the DNA base sequence for
transcription, a suitable cDNA or genomic DNA or synthetic
polynucleotide may be used. The isolation of suitable ATH1
sequences from Arabidopsis is described in Quaedvlieg et
al., above: similar methods may be used to isolate ATH1
25 sequences from other plants. These may have greater or
lesser degrees of homology with ATH1 sequences from
Arabidopsis. Sequences coding for the whole, or
substantially the whole, of the protein may thus be
obtained. Suitable lengths of this DNA sequences may be
30 cut out for use by means of restriction enzymes. When
using genomic DNA as the source of a partial base sequence
for transcription it is possible to use either intron or
exon regions or a combination of both.

To obtain constructs suitable for modifying expression of ATH1 in plant cells, the cDNA sequence as found in the protein cDNA or the gene sequence as found in the chromosome of the plant may be used. Recombinant DNA

5   constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by

10 annealing and ligating synthetic oligonucleotides or by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator

15 sequences. If antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

20   In a construct expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and vice versa. The construct will thus encode RNA in a base sequence which is complementary to part or all of the sequence of the protein mRNA. Thus the

25 two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

          In a construct expressing sense RNA, the template and coding strands retain the assignments and

30 orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence which is homologous to part or all of the sequence of the mRNA. In constructs which express the functional protein, the whole of the coding region of the gene is linked to

transcriptional control sequences capable of expression in
plants.

For example, constructs according to the
present invention may be made as follows. A suitable
vector containing the desired base sequence for
transcription (such as the pATH1 cDNA clone) is treated
with restriction enzymes to cut the sequence out. The DNA
strand so obtained is cloned (if desired, in reverse
orientation) into a second vector containing the desired
promoter sequence and the desired terminator sequence.
Suitable promoters include the 35S cauliflower mosaic virus
promoter and the tomato polygalacturonase gene promoter
sequence (Bird et al, 1988, Plant Molecular Biology,
11:651-662) or other developmentally regulated plant
promoters. Suitable terminator sequences include that of
the Agrobacterium tumefaciens nopaline synthase gene (the
nos 3' end).

In a DNA construct according to the invention,
the transcriptional initiation region may be derived from
any plant-operative promoter. The transcriptional
initiation region may be positioned for transcription of a
DNA sequence encoding RNA which is complementary to a
substantial run of bases in a mRNA encoding the ATH1
protein (making the DNA construct a full or partial
antisense construct).

The transcriptional initiation region (or
promoter) operative in plants may be a constitutive
promoter (such as the 35S cauliflower mosaic virus
promoter) or an inducible or developmentally regulated
promoter, as circumstances require. For example, it may be
desirable to modify protein activity at certain stages of
the plant's development. Use of a constitutive promoter

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will tend to affect protein levels and functions in all parts of the plant, while use of a tissue-specific promoter allows more selective control of gene expression and affected functions. Thus the antisense or sense RNA is only
5 produced in the organ in which its action is required.

The DNA constructs of the invention may be inserted into plants to regulate the expression of the ATH1 gene resulting in modification of plant characteristics (in
10 particular flowering). Depending on the nature of the construct, the production of the ATH1 gene product may be increased, or reduced, either throughout or at particular stages in the life of the plant. Generally, as would be expected, production of the protein is enhanced only by
15 constructs which express RNA homologous to the substantially complete endogenous protein mRNAs. Full-length sense constructs may also inhibit protein expression. Constructs containing an incomplete DNA sequence shorter than that corresponding to the complete
20 gene generally inhibit the expression of the gene and production of the proteins, whether they are arranged to express sense or antisense RNA.

A DNA construct of the invention is transformed
25 into a target plant cell. The target plant cell may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. The target plant cell may be selected from any monocotyledonous or dicotyledonous plant species. Plants may be derived
30 from the transformed plant cell by regeneration of transformants and by production of successive generations of the transformants' progeny.

Constructs according to the invention may be
35 used to transform any plant using any suitable

transformation technique to make plants according to the
invention. Both monocotyledonous and dicotyledonous plant
cells may be transformed in various ways known to the art.
In many cases such plant cells (particularly when they are
5   cells of dicotyledonous plants) may be cultured to
regenerate whole plants which subsequently reproduce to
give successive generations of genetically modified plants.
Any suitable method of plant transformation may be used.
For example, dicotyledonous plants such as tomato and melon
10   may be transformed by *Agrobacterium* Ti plasmid technology,
such as described by Bevan (1984, Nucleic Acid Research,
12:8711-8721) or Fillatti et al (Biotechnology, July 1987,
5:726-730). Such transformed plants may be reproduced
sexually, or by cell or tissue culture. Monocots may be
15   transformed by use of the gene gun. Other methods for plant
transformation include microinjection and electroporation.

Examples of genetically modified plants according to the
present invention include cereals, for example rice and
20   maize, wheat, barley, oats and rye. Other important seed
products are oilseed rape (canola), sugar beet, sunflower,
soya and sorghum. Most crops are grown annually from seed
and the production of seed of any kind depends upon the
ability of the plant to flower, to be pollinated and to set
25   seed. In horticulture, control of the timing of flowering
is important. Horticultural plants whose flowering may be
controlled include lettuce, endive and vegetable brassicas
including cabbage, broccoli and cauliflower, and carnations
and geraniums.
30

The main characteristics of modified plants according to
the invention are early or delayed flowering. Genotypes in
which production of the ATH1 protein is inhibited generally
flower early: genotypes in which it is stimulated flower

late. Other effects on plant phenotype may also be
observed, e.g. dwarf habit, for example in tobacco.

Control of the time of flowering may be useful for several
5   reasons. For example, flowering may be controlled to
provide flowers or fruit at the time most appropriate for
marketing. In hybrid production, flowering of male and
female parents may be co-ordinated. It is most convenient
to do this by the use of inducible gene promoters,
10   responsive to external stimuli, for example application of
chemicals. An example of such a promoter is the maize
glutathione-S-transferase isoform II gene promoter,
activated by application of a known herbicide safening
agent (WO93/01294 to ICI).
15

Bolting control may be economically important in several
crop species. For example, in sugarbeet, producing
varieties which have a reduced tendency to bolt after cold
treatment would be of great use. Processing factories
20   could spread their activities over a longer period of time,
with significant savings in overheads. Bolting-resistant
varieties could be sown very early in the season
(February) or even the year before in autumn (provided
winter frost was not a problem). Further, varieties in
25   which bolting is increased may be bred faster: crossings
may be carried out annually instead of biannually as at
present.

Early flowering sunflower would have an extended
30   geographical range. It could be grown further north (north
of Paris), and possibly in drier regions, e.g. parts of
Spain, avoiding periods of drought later in summer.

In vegetables, bolting may be controlled in for example
35   lettuce and endive. This would allow growing the crop more

easily during summer. Existing varieties tend to bolt
rather rapidly under summer conditions. In grasses, reduced
(or no) bolting is beneficial for fodder types (improved
feed quality) and amenity types (better quality lawns).

It will on occasion be of advantage to time the expression
of transgenes to stop when flowering starts, or suppress
naturally-occurring genes until flowering starts. This may
be done using the ATH1 promoter to control expression of a
transgene, or transcription of DNA homologous to a natural
gene. Accordingly it is a further separate feature of the
invention to provide a DNA construct comprising the ATH1
promoter linked to heterologous DNA so as to cause
transcription thereof in plant cells: and plant cells
transformed with such DNA constructs.

ATH1 is expressed in the vegetative apical meristem, and
downregulation of this expression coincides with floral
transition. Forced constitutive expression of ATH1 results
in a dramatic repression of floral transition both in
Arabidopsis and tobacco: thus, in the case of *Arabidopsis*
bolting is postponed. Conversely, repression of ATH1
results in an early flowering phenotype. Our results
suggest that ATH1 exerts its function through modulation of
GA biosynthesis or responsiveness. We expect the ATH1 gene
to be the basis of a particularly useful bolting control
system.

Day length and floral transition

The floral transition has been particularly well
investigated in *Arabidopsis thaliana*. This species has
become the model system for studying floral transition: at
the genetic level through the isolation of flowering-time
mutants; and at the molecular level through cloning of

genes whose products participate in the control of floral
transition. *Arabidopsis* is a typical rosette plant in which
the vegetative leaves are closely spaced due to reduced
internode elongation. Upon floral transition the newly
5   formed internodes rapidly elongate ('bolting'). In most
Arabidopsis ecotypes day length is of major importance in
determining floral transition. *Arabidopsis* is a
facultative long day (LD) plant, which means that floral
transition is hastened by long days (16 hours light/8hours
10   dark cycle), but there is no obligate requirement for it.
Under long day (LD) conditions floral transition is rapidly
initiated and only a few rosette leaves are formed (~7
leaves, 16-20 days for the Col-0 ecotype). When grown
under short days (SD), e.g. 8 hrs light/16 hrs dark, floral
15   transition takes much longer (~60 days) and a full leafy
rosette is formed which can have in excess of ~30 rosette
leaves (Col-0 ecotype).

Gibberellic acid (GA) and floral transition
20
It has been known for a long time that GA treatment
promotes floral transition in a variety of plant species.
Most species in which applied GA can induce flowering are
long-day or cold-requiring plants, and many of these
25   normally grow as rosettes under non-inductive conditions.
Moreover, several experiments suggest that endogenous GA
levels are involved in controlling floral transition:
conditions that induce floral transitions can exert their
effect through elevation of endogenous GA levels probably
30   at or near the apical meristem.

Arabidopsis mutants defective in GA biosynthesis (GA
series) or insensitive to this hormone (GAI series) show a
late flowering phenotype under non-inductive conditions and
35   moreover, the severe GA1-3 mutant is also late flowering
under inductive conditions (Wilson et al., 1992).

Involvement of GA in ATH1 control of floral transition

We tested whether exogenous GA can overcome the inhibitory
5   effect of constitutive ATH1 expression in tobacco. Most
remarkably, GA spraying was able to 'rescue' the late
flowering phenotype in constitutive ATH1 expressers in
tobacco. An involvement of GA was also indicated by the
reduced internode elongation phenotype in the tobacco ATH1
10   expressers. These findings suggest that ATH1 functions as
a repressor of GA biosynthesis or, alternatively, of GA
responsiveness. The dominant effect of ATH1 over-
expression on floral transition in combination with the
reversion of this effect by exogenously added GA suggests
15   several uses in a variety of crops. This is especially
interesting since deregulated expression of ATH1 does not
lead to pleiotropic phenotypes and reversion of the
overexpression phenotype is complete. Complete rescue
means that there will be no problems regarding reproduction
20   or multiplication of ATH1-transformants: thus maintaining
the transgenic lines, which can be a serious problem with
flowering mutants, is straitforward. Using the GA switch,
plants can be reversed to wild-type development at any
moment, plants flower normally, and there is a normal seed
25   set.

Accordingly, it is a further feature of the invention to
inhibit over-expression of ATH1 in plants genetically
modified according to the invention by treating the plants
30   with a gibberellin, for example gibberellin A3 or A4/A7.

Increase of harvest index

When plants grow in close proximity, shade-avoidance
35   syndrome, in which plants react to far-red radiation

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reflected from neighbors, is manifested. This most
obviously results in a rapid and dramatic increase in the
extension growth of stems and petioles at the expense of
leaf growth, storage organ production, and reproductive
5 development. It is known that by overexpression of phyA
genes in tobacco the shade-avoidance response can be
overcome, resulting in an increased harvest index (Robson
et al., 1996). Harvest index is expressed as leaf biomass
as a proportion of total biomass. Overexpression of ATH1
10 in tobacco causes a reduction in stem growth, while leaf
growth and number stay unaffected or even increase compared
to wild type. As ATH1 overexpression phenotypes and phyA
overexpression phenotypes are similar, this suggests using
ATH1 overexpression to increase harvest index in crops.
15 The invention will be further described with reference to
the following Examples and Experiments, which illustrate
certain aspects of our invention: and with respect to the
drawings, in which:

20   Figure 1 gives the DNA sequence of ATH1 cDNA;
            Figure 2 is a diagram of the plasmid pWP90;
            Figure 3 is a diagram of the plasmid pMOG23;
            Figure 4 is a diagram of the plasmid pVDH275;
            Figure 5 is a bar graph showing the dwarfing caused
25 by constitutive expression of ATH1 in 90 days old tobacco
plants;
            Figure 6 is a graph showing the effect of
gibberellin treatment on the height of tobacco plants
overexpressing ATH1;
30   Figure 7 is a bar graph showing flowering time (in
terms of number of rosette leaves formed) for under- and
over-expressors of ATH1 in comparison with wild-type
Arabidopsis (C24 ecotype).

35 **General Methods**

Plant material and plant growth conditions

The wild-type genotypes used were *Arabidopsis thaliana*
Columbia and C24. The ATH1 gene is located on chromosome 4,
between the RFLP markers mi431 (96.9 cM) and C6455 (97.9
cM). *Arabidopsis thaliana* Columbia was used in plant
transformation experiments using the vacuum infiltration
protocol, while *Arabidopsis thaliana* C24 was used in plant
transformation experiments using the root transformation
protocol.

Plants were grown in a growth chamber under fluorescent
light with a photoperiod of 16 hours followed by an 8 hours
dark period at a continuous temperature of 22°C.

To measure flowering time seeds were imbibed and placed at
4°C for 4 days to break dormancy and were then sown on
soil. Germinating seedlings were usually covered with
propagator lids for the first 1-2 weeks to prevent
dehydration.

Transformation of *Arabidopsis* plants

Binary constructs containing chimeric ATH1-GUS genes and
35S-antisense ATH1 genes were transformed into *Arabidopsis
thaliana* ecotype C24 using the *Agrobacterium tumefaciens*-
mediated root transformation method of Valvekens et al.
(1988). Transformants were selected on medium containing 50
mg/l kanamycin.

Binary constructs containing chimeric 35S-ATH1 genes were
transformed into *Arabidopsis thaliana* ecotype Columbia
using the vacuum infiltration protocol (Bent et al. (1994);
Bechtold et al. (1993)) with some modifications. Plants

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were grown separately in 5.5 cm pots. Plants were transformed after appearance of the first siliques on the secondary bolts.

5   900 ml cultures of *Agrobacterium tumefaciens* containing the appropriate construct were grown the night before the day of infiltration, cells were harvested by centrifugation and resuspended in an equal volume of infiltration medium, containing 2% instead of 5% sucrose. Plants were
10 infiltrated by submerging entire rosettes and bolts for 10 minutes under a vacuum pressure of 100mm Hg.

Transformant seeds were selected on medium containing 50 mg/l kanamycin.
15

EXAMPLE 1

ATH1 expression analysis

20 **Total RNA isolation**

Total RNA from plants was isolated according to De Vries et al. (1988) with some minor modifications: (1) plant tissue was ground in liquid nitrogen in the presence of half the
25 volume of phenol/extraction buffer and heated to 65°C in a water-bath and (2) the RNA was ethanol/Na-acetate precipitated before and after LiCl precipitation.

RNAase protection analysis
30
The HindIII-XhoI fragment of phagemid ATH1 was cloned into pBluescriptSK(-) (Stratagene) and digested with HindIII to produce a T7 RNA polymerase template. The ATH1 RNA probe protects a fragment of 140 nt. RNA probe was synthesized
35 by using T7 RNA polymerase (Pharmacia) and buffer as

described by the manufacturer, except that 160 μCi of [-
$_{32}$P]UTP (800 Ci/mmol) was used. RNAase protection was done
by using 10 μg of total RNA and 10μg of tRNA according to
the protocol described by Sambrook et al. (1989). The

5   digested mixture contained 600 units/ml RNAase T1 (Gibco
BRL) and 20 μg/ml RNAase A (Boehringer). RNA:RNA hybrids
were analyzed by sequence gel electrophoresis (6%
polyacrylamide/ 7M urea) and visualized by autoradiography.

10   **Construction of chimeric ATH1-GUS constructs**

A SpeI-NcoI fragment containing approximately 1300
nucleotides of ATH1 promoter sequence was isolated. After
filling in the *NcoI* site with Klenow-polymerase, this

15   fragment was inserted into the unique *SmaI/XbaI* sites of
the pBi101.1 binary vector which contains the GUS gene
(Jefferson et al., 1987), creating a translational fusion
between the ATH1 promoter and the GUS gene. The protein
encoded by this chimeric gene consists of 42 aa of ATH1

20   fused to the GUS protein. The binary construct was called
tH1.4. tH1.4 was transformed into competent *Agrobacterium
tumefaciens* LBA4404 cells (Gelvin and Schilperoort, 1988).
Arabidopsis lines (ecotype C24) were transformed as
described below.

25

In situ **localization of GUS activity in transgenic ATH1-GUS**
Arabidopsis thaliana **lines**

Seedlings and plant tissues were collected and stained for

30   1 to 16 hours at 37°C in a solution containing 0.5 mg/ml X-
Gluc (Biosynth AG) dissolved in n-dimethyl-formamide, 0,1%
Triton X-100, 0.5 mM K4Fe(CN)6.H2O, 0.5 mM K3Fe(CN)6 and 50
mM sodium phosphate buffer, pH 7.2.

35   **Light microscopy**

After X-Gluc staining, plant tissues were fixed overnight
in a solution containing 1% glutaraldehyde and 4%
formaldehyde in 50mM sodium phosphate buffer, pH 7.2.
5   Subsequently seedlings were dehydrated in gradual steps:
10%, 30%, 50%, 70%, 90% and 2x 100% ethanol. Large plant
tissues were pre-embedded first in 1% agarose (Sigma).
Infiltration and embedding in Technovite 7100 (Kulzer,
Hereaus) was performed as instructed by the manufacturer. 4
10   um sections were made on a Reichert-Jung 1140 rotary
carrying a disposable Adams steel knife. Sections were
stained with 0.1% Ruthenium red (Sigma) in distilled water
for 2 minutes at room temperature and photographed on a
Zeiss Axioskop using Kodak Professional Ektar 25 film.
15

Seedlings were fixed and dehydrated as above. Technovit
7100 was infiltrated for 1 day. The seedlings were then
transferred to a construction of celluloid transparency
(Amovis), double-sided tape, transparency, double-sided
20   tape. In the latter three layers a central region was
excised to contain the seedling. Subsequently the
seedlings were added in Technovit 7100 solution and the
central region was covered by another transparency. Upon
overnight polymerisation at room temperature a plastic
25   platelet containing the seedling was obtained. In order to
section embedded seedlings in the platelet, the celluloid
sheet material was removed and the platelet was cut to
allow longitudinal sectioning of relevant seedling regions.
Sectioning, staining and photographing was performed as
30   described above.

Localization of ATH1 expression
   The expression of the ATH1 gene was analyzed using RNA-ase
protection analysis (Quaedvlieg et al., 1995). High levels
35   of ATH1 mRNA were detected during early seedling

development (days 2-6) and in flowers of mature *Arabidopsis*
plants. The cellular localization of ATH1 gene expression
was determined by introduction of the chimeric ATH1-GUS
construct tH1.4 in *Arabidopsis thaliana*. Different tissues
were stained with X-gluc, and whole mount preparations and
tissue sectioning were made to visualize GUS activity (see
below).

ATH1 expression during vegetative development

The shoot apex of a 5-day-old light-grown seedling is flat
and consists of a two-layered tunica enclosing the
subjacent corpus. At this stage, the meristem has initiated
the primordia of the first leaf pair (Mischke and Brown,
1965).

In plants transformed with tH1.4, high levels of GUS
activity were present in the shoot apex. Sectioning of the
shoot apex showed that the high GUS activity is shown in
all three layers of the shoot apical meristem and extends
through the subapical region, proceeding down to where the
vascular strand of the hypocotyl branches into the
cotyledons. High levels of GUS activity were also present
in the primordia of the first leaf pair.

**ATH1 expression during floral transition and inflorescence
development**

Initially, during the inflorescence phase, the shoot apical
meristem gives rise to stem, cauline leaves and secondary
inflorescences. As inflorescence development proceeds, the
inflorescence meristem produces flower primordia. In plants
transformed with tH1.4, GUS activity was downregulated in
the inflorescence meristem during the transition phase.
There was no GUS activity detectable in the meristem. Low

levels of GUS activity were present in the rib zone. Later
when flowers arose, GUS activity was present in different
parts of the young flower (receptacle, sepals and vascular
tissue of stamen)

EXAMPLE 2

Construction of promoter fusions to the ATH1 open reading frame

The ATH1 cDNA is cloned into the unique EcoRI/XhoI
restriction sites of the well-known and commercially
available pBluescript SK(-) vector (Stratagene).

2.1. A CaMV 35S promoter fusion to the ATH1 open reading frame

A BamHI/SnaBI fragment containing 1573 nuclectides of ATH1
cDNA sequence (the BamHI site was created by PCR
mutagenesis, 35 nucleotides downstream of the translation
start) was isolated and inserted into the unique BamHI/SmaI
cloning sites of pWP90-vector, which contains a double 35S
CaMV promoter and a NOS terminator (see Figure 2),
resulting in a transcriptional fusion between the double
35S CaMV promoter and ATH1 cDNA. This construct, called
cH1.24, was then cut with SstI/EcoRV restriction enzymes,
followed by insertion of the resulting SstI/EcoRV insert in
the unique SstI/SmaI restriction sites of binary vector
pMOG23 (see Figure 3). The binary construct was called
tH1.2. tH1.2 was transformed into competent *Agrobacterium
tumefaciens* pGV2260 cells (Caplan *et al*., 1985) cells.
Arabidopsis lines (ecotype Col-0) were transformed via
vacuum infiltration as described below

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2.2 Construction of a CaMV 35S promoter fusion to the antisense ATH1 frame

5 An EcoRI/SnaBI fragment containing approximately 1330 nucleotides of ATH1 cDNA sequence was isolated and inserted into the unique SmaI/EcoRI cloning sites of pWP90 vector (see Figure 2), resulting in a transcriptional fusion between the

10 double CaMV 35S promoter and the antisense ATH1 frame. The resulting construct was called cH1.22. An EcoRV/SstI insert of cH1.22 was then cloned into the unique SmaI/SacI restriction sites of the binary vector pMOG23 (MOGEN)(see Figure 3).

15 This binary construct, called tH1.1, was transformed into competent *Agrobacterium tumefaciens* LBA4404 cells. *Arabidopsis* lines (ecotype C24) were transformed as described below.

20

2.3. A heat shock promoter fusion to the ATH1 open reading frame

By PCR mutagenesis, an additional BamHI site was
25 created in pTT19, a vector containing the promoter, leader and 77 nucleotides of coding sequence of the *Arabidopsis thaliana* Hsp18.2 heat shock gene (Takahashi and Komeda, 1989). The additional BamHI site is located in the Hsp18.2
30 untranslated leader at nucleotide -710 of the Hsp18.2 translational start.

By restriction digestion with BamHI the 5' untranslated leader and 77 nucleotides of Hsp18.2
35 coding sequence were removed. The remaining

construct was called leaderless pTT19. A
HindIII/BamHI fragment of this leaderless pTT19,
containing only Hsp18.2 promoter sequence, was
fused to a BamHI/EcoRI fragment containing the
5   entire ATH1 cDNA sequence, which results in a
transcriptional fusion of Hsp18.2 promoter with
ATH1 5' untranslated leader and coding sequence.
The BamHI and EcoRI sites were created by PCR
mutagenesis, resulting in a BamHI restriction
10   site at the beginning of the ATH1 cDNA sequence
and a EcoRI restriction site immediately
downstream of the TAA stop codon. The resulting
HindIII/EcoRI fragment was inserted into the
unique HindIII/EcoRI restriction sites of pWP90
15   vector. (see Figure 2) and this new construct was
then partially digested with HindIII and EcoRV
restriction enzymes. The largest HindIII/EcoRV
restriction fragment was then inserted into
HindIII/SmaI cut binary vector pBIN 19 (Frisch et
20   al., 1995). This construct was called HspH1.

A transcriptional fusion between Hsp18.2 promoter
and ATH1 coding sequence without leader sequence
was also made. In ATH1 cDNA an extra BamHI site
25   was created by PCR mutagenesis immediately
upstream of the translational start. Digestion of
this BamHI site combined with digestion of the
unique XhoI site in ATH1 cDNA results in an
fragment of approximately 680 nt, containing ATH1
30   coding sequence. This fragment of 680 nucleotides
was swapped with an approximately 980 nucleotides
large fragment that is formed after digestion of
HspH1 with BamHI/XhoI restriction enzymes. This
results in HspH1B, a transcriptional fusion
35   between leaderless ATH1 coding sequence and the

Hsp18.2 promoter.

Both HspH1 and HspH1B were transformed to competent
Agrobacterium tumefaciens LBA4404 cells. *Arabidopsis* lines
5   (C24 ecotype) were transformed as described below.

2.4 Fusion of the pea plastocyanin promoter to the ATH1 open reading frame

10   A transcriptional fusion between pea plastocyanin
     promoter and ATH1 coding sequence can be made by
     insertion of ATH1 coding sequence into the unique
     BamHI and SalI restriction sites of pVDH275 (Pwee
     and Gray, 1993; Last and Gray, 1989) (see also
15   Figure 4). In ATH1 coding sequence additional
     SalI (immediately upstream of ATH1 start ATG) and
     BamHI (immediately after ATH1 stop TAA)
     restriction sites can be created by PCR
     mutagenesis. The resulting construct in which
20   ATH1 coding sequence is inserted between pea
     plastocyanin promoter and *Agrobacterium* nos
     terminator, can be transformed to *Agrobacterium*
     tumefaciens cells, followed by plant
     transformation.
25

Introduction of extra ATH1 copies in *Arabidopsis*

     Extra copies of ATH1 can be introduced in
     Arabidopsis plants by transforming them with
30   extra ATH1 loci containing ATH1 promoter and ATH1
     coding sequence. This can be done by fusion of
     the approximately 1000 nucleotides large
     SnaBI/NcoI fragment of ATH1 cDNA to the
     approximately 250 nucleotides large SstI/EcoRI
35   restriction fragment of pBI101.1, containing the

Agrobacterium nos terminator (Jefferson et al.,
1987). The resulting fragment can be fused to the
approximately 3.5 Kb large NcoI restriction
fragment of ATH1 genomic clone (Quaedvlieg et
5   al., 1995). The so formed approximately 4750
nucleotides large NcoI/EcoRI fragment, containing
ATH1 promoter, ATH1 coding sequence and nos
terminator, can be inserted into NcoI/EcoRI cut
pMTL23 cloning vector (Chambers et al., 1988). A
10   StuI/EcoRI restriction fragment of the resulting
construct can then be inserted into EcoRI/SmaI
cut pMOG23 binary vector, *Agrobacterium* cells can
be transformed, subsequently followed by plant
transformation.
15

EXAMPLE 3

**Influencing flowering characteristics using a CaMV 35S
promoter/ATH1 gene fusion**

20   **Measurement of flowering time**

Flowering time was measured by counting the number of
leaves, excluding the cotyledons, in the rosette at the
time the flower bud was visible. A close correlation
25   between leaf number and flowering time has been previously
demonstrated (Koorneef et al., 1991; Bagnall (1993)).

Overexpression of ATH1 leads to delayed flowering.

30   In order to gain more insight into the role of ATH1 in
plant development, the full length ATH1 cDNA sequence was
fused to the constitutive 35S promoter of cauliflower
mosaic virus and the 35S::ATH1 chimeric gene so produced
was transformed into *Arabidopsis* Col-0 ecotype via the

vacuum infiltration method. Six independent primary
transformants were obtained.

All these transgenic lines were selfed. From each
5 independent transgenic line 40 individual seeds were
germinated on soil and scored for altered phenotypes
compared to wild-type plants. Four out of six lines showed
a phenotype altered in respect of flowering time. In
three of these lines all plants were late flowering (about
10 14 rosette leaves up to flowering compared with about 10
rosette leaves in wild-type Col-0 plants). In the
remaining line about 85 % of the plants showed this same
late flowering phenotype, while 15 % of the plants showed
an early flowering phenotype (after about 7 rosette
15 leaves), as tested due to the absence of ATH1 RNA. These
early flowering plants also show a terminal flower
phenotype, often with incomplete flowers and mutant flower
organs.

20   **EXAMPLE 4**

Early flowering by antisense expression of ATH1
Like ectopic overexpression of ATH1, inhibiting the ATH1
gene function can also can be used to influence time of
25 flowering. Inhibition of gene function was effected by
constitutive overexpression of antisense ATH1.

Full length antisense ATH1 cDNA was fused to the
constitutive 35S promoter of cauliflower mosaic virus and
30 the 35S::antisense ATH1 chimeric gene so produced was
transformed into *Arabidopsis* C24 ecotype via the Valvekens
root transformation protocol. Twenty-two independent
transformants were obtained and all of them were selfed.
From each line 10 individual seeds were germinated on soil
35 and scored for altered phenotypes compared to wild-type C24

plants. In five of these lines, the plants showed an early
flowering phenotype: flowering started after formation of
between six and ten rosette leaves compared to about twenty
leaves in wild-type plants.

5

EXAMPLE 5

Altering flowering time in *Nicotiana tabacum* by

10   overexpression of ATH1

As in *Arabidopsis*, ectopic overexpression of ATH1 cDNA
(driven by the 35S promoter of cauliflower mosaic virus)
in tobacco (*Nicotiana tabacum* cv. Samsun) also led to a

15   delay in flowering time compared to wild-type tobacco. In
35S::ATH1 tobacco plants, flowering was delayed by weeks or
months. These plants were also dwarfed. This dwarf habit,
like the flowering phenotype, is clearly correlated with
the level of expression of the transgene. In the severest

20   case plants did not flower at all and only reached one-
fifth of their normal height, whereas in less severe cases
plants were delayed in flowering for only one or two weeks
and reached about four-fifth of their normal height. Leaf
number and shape were normal in all these transformed

25   plants.

EXAMPLES 6-8

The following Examples illustrate the effect of GA on
transgenic plants according to the invention. As noted

30   above, ATH1 overexpression effectively represses bolting
(floral induction). We hypothesised that ATH1 may be a
repressor of GA synthesis or the GA response pathway (we
think the former). The following Examples demonstrate and
support this hypothesis.

35

General Methods

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were
transformed using the leaf disk procedure (Horsch et al.,
5   1985). Transgenic plants were selected on MS-medium
(Murashige and Skoog, 1962) containing 300 mg/ml kanamycin
and 2% sucrose. After transfer to soil plants were grown
in a greenhouse at 22 °C under a light regime of 16 hours
daylight when necessary supplemented with artificial light.
10   The effects of gibberellin (GA) were tested by foliar
applications (spraying) of 100mM GA3 in a solution
containing 100ml/1 of Triton X-100. Control plants were
sprayed with a solution containing only 100ml/1 of Triton
X-100. Spraying began 60 days after sowing when the wild-
15   type plants were approximately 5 cm tall and the 35S
CaMV::ATH1 plants approximately 2.5 cm tall, and continued
at 3- to 4- day intervals. Plant height was measured every
3 to 4 days and this will be continued until the onset of
flowering, as determined by the appearance of flower
20   primordia.

EXAMPLE 6

Constitutive overexpression of sense ATH1 leads to delayed
flowering.

25

6.1 ATH1 over-expression in tobacco

In order to express the ATH1 gene constitutively in
transgenic plants, its coding region was put under the
30   control of the 35S CaMV promoter and the resulting
construct was transformed to tobacco (*Nicotiana tabacum* cv.
Samsun NN). Forty independent kanamycin-resistant plants
were obtained, of which only five showed detectable
transgene expression. ATH1 mRNA levels varied from high in
35   H1CE#4, #10 and #30 plants to intermediate/low levels in

H1OE#35 and #37 plants. Depending on ATH1expression level,
flowering of these plants was delayed by weeks up to
months, when compared to wild-type plants, which flower
after 3-5 months depending on the season. In the severest
5   case (H1OE#4) plants never flowered until senescence (>15
months after sowing). H1OE#10 and #30 plants, which show
high ATH1 expression, flowered after 15 months, while
plants showing the intermediate/low overexpression, H1OE#35
and 37, did not flower until after 6 months. As well as
10 altered flowering-time phenotype, ATH1 overexpressor plants
show reduced stem growth, resulting in dwarfed plants. Here
there is a clear correlation between severity of the dwarf
growth phenotype and the level of transgene expression (see
Figure 5). In the severest case plants only reach about
15 one-fifth of their normal height. The leaf number varies
from two times higher than wild type to normal in all
transgenes.

6.2 ATH1 overexpression can be reversed by GA3

20
ATH1 overexpression phenotypes can be reversed to a wild-
type phenotype by application of GA3. Foliar application
of GA3 to the tobacco plants of Example 6.1 (spraying of
100 mM GA3 at three to four day intervals) results in
25 complete restoration of the wild-type stem length (Figure
6). This holds also true for the late-flowering
phenotype (data not shown).

EXAMPLE 7

30
ATH1 over-expression in *Arabidopsis*

In order to gain more insight into the role of ATH1 in
plant development, the full length ATH1 cDNA was fused to
35 the constitutive 35S promoter of cauliflower mosaic virus

and the 35S::ATH1 chimeric gene was transformed into
Arabidopsis via the vacuum infiltration method. Six
independent primary transformants could be obtained and all
transgenic lines were selfed. From each independent
5   transgenic line 40 individual seeds were germinated and
scored for altered phenotypes compared to wild type plants.
Four out of six lines showed an altered phenotype in
respect of flowering time. Seeds from these lines did not
germinate well and if they did plants were arrested in a
10 seedling stage. Both effects could be overcome by
transferring the plants to growth medium containing 10-5 M
GA3 and growing them on this medium for three days. Once
rescued and transferred to soil plants developed normally,
except for a late flowering phenotype. Under short day
15 conditions transgenic plants form much more rosette leaves
(vegetative leaves) than wild type plants (about 40 rosette
leaves and 100 days after germination, and plants are still
not flowering, compared to about 30 rosette leaves in wild-
type plants until flowering). Under LD conditions in most
20 of these plants a partial generative to vegetative
reversion occurs, shown by the formation of aerial rosettes
(vegetative leaves) on the inflorescence stem.   Plants
(C24 ecotype) containing an extra copy of the ATH1 cDNA
under control of the Hsp18.2 heat shock promoter (HspH1B
25 plants) also show a late-flowering phenotype. Even without
a heat shock (it is known that this promoter has a basal
activity without induction) plants harboring this construct
flower much later under LD conditions than wild-type plants
(30.5 rosette leaves formed in wild-type vs. 61 rosette
30 leaves formed in HspH1B plants - see Figure 7).

EXAMPLE 8

Early flowering by antisense expression of ATH1

35

Like ectopic overexpression of ATH1, knocking out the ATH1 gene function can also give insights into the function of ATH1 in plant development. Knocking out gene function was established by constitutive overexpression of antisense

5   ATH1. Full length antisense ATH1 cDNA was fused to the constitutive 35S promoter of cauliflower mosaic virus and the 35S::antisense ATH1 chimeric gene was transformed into *Arabidopsis* C24 ecotype via the Valvekens root transformation protocol. Twenty-two independent

10   transformants were obtained and all of them were selfed. From each line 10 individual seeds were germinated on soil and scored for altered phenotypes compared to wild type C24 plants. In five of these lines plants showed an early-flowering phenotype: flowering started after formation of

15   about ten rosette leaves compared to about thirty leaves in wild type plants (see Figure 7).

Example 9

Shade avoidance response

20   When plants grow in close proximity shade avoidance syndrome, in which plants react to lowered red/far-red ratios of light caused by filtering out red light by leaf canopy, is manifested. This results in a rapid and dramatic increase in the extension growth of stems and petioles at

25   the expense of leaf growth, storage organ production, and reproductive development, thereby causing a decrease in harvest index (where harvest index is expressed as leaf biomass as a proportion of total biomass).

The shade avoidance response is thought to be predominantly

30   mediated by phytochrome B and overexpression of phytochrome has been shown to eliminate the shade avoidance response, resulting in an increase of harvest index of field-grown tobacco (Robson *et al.*, 1996).

Lack of phytochrome B also leads to loss of shade avoidance

35   response and under inductive conditions this even results

in a reduction of stem elongation compared with non-inductive conditions.

In the laboratory, situations causing the shade avoidance response can mimicked by addition of different fluence

5   rates of far-red light (Frc) to continuous white light (Wc). Under these conditions wild-type *Arabidopsis* plants (C24 wt) show a typical shade avoidance response (elongated hypocotyls in Wc + Frc compared with hypocotyl length in Wc only), whereas the phytochrome B photoreceptor mutants,

10   which lack the shade avoidance response, exhibited an opposite response (decrease of hypocotyl length). The antisense AtH1 plants also show a reduction in hypocotyl length and in the most severe antisense plants (asAtH1#3) this reduction is similar to that seed in phyB mutants. So,

15   it can be concluded that like lack of active phytochrome B loss of Ath1 results in loss of the shade avoidance response.

Shade avoidance analysis of antisense AtH1 plants (asAtH1), C24 wild-type plants (C24 wt) and the phy B photoreceptor

20   mutant (phyB) is listed below. Seedlings where grown for 2 days in continuous white light followed by 4 days in the same light regime or by 4 days in white light supplemented by far-red light. The hypcototyl length was measured after 6 days of growth. The following results were generated.

25

C24wt	Wc	= hypocotyl length	5.5 mm
	Wc + Frc	= hypocotyl length	7.5 mm
Phy B	Wc	= hypocotyl length	9.5 mm
	Wc + Frc	= hypocotyl length	7.3 mm
Anti-sense AtH1#3	Wc	= hypocotyl length	9.0 mm
	Wc + Frc	= hypocotyl length	5.0 mm
Anti-sense	Wc	= hypocotyl length	9.8 mm

```
AtH1#7       Wc + Frc   =   hypocotyl length   8.0 mm


Anti-sense   Wc         =   hypocotyl length   7.5 mm
AtH1#23      Wc + Frc   =   hypocotyl length   7.0 mm
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